

PATENT
Docket No.: 176/61481 (Goldfarb)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : David S. Goldfarb) Examiner:
Serial No. : 10/790,456) Walter Schlapkohl
Cnfrm. No. : 9599) Art Unit:
Filed : March 1, 2004) 1636
For : MATERIALS AND METHODS FOR)
IDENTIFYING GENES AND/OR AGENTS)
THAT ALTER REPLICATIVE LIFESPAN)

DECLARATION OF DAVID S. GOLDFARB UNDER 37 CFR § 1.132

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, David S. Goldfarb, pursuant to 37 C.F.R. § 1.132, hereby declare:

1. I am an inventor of the above-identified application.
 2. I am currently a Professor in the Department of Biology at the University of Rochester, Rochester, NY. I have served in that capacity since July 1998. From April 1988 to April 1993, I was an Assistant Professor in the Department of Biology at the University of Rochester. From May 1993 to June 1998, I was an Associate Professor.
 3. I received a Ph.D. in Biochemistry in 1983 from the University of California at Davis. I received a B.A. in Biology in 1977 from the University of California at San Diego. My research interests include: nuclear transport, nuclear envelope, autophagy, lifespan, and stress response.
 4. I am presenting this declaration to (i) demonstrate that the study of yeast replicative lifespan is relevant to the physiology of aging in higher animals, and (ii) submit

additional evidence demonstrating that the screening assay of the present invention faithfully reproduces important features of the standard yeast replicative lifespan. For these reasons, the methods (assays) as claimed in the present invention represent a valid approach for identifying environmental and genetic modifications that may alter lifespan in both yeast and higher animals.

Yeast Replicative Lifespan is Relevant to the Physiology of Lifespan in Higher Animals

5. The present application describes a novel replicative lifespan assay in yeast, and methods for using the assay to identify environmental stimuli or genetic modifications which alter lifespan. As set forth in paragraphs 6-9 below, yeast are art-accepted models for the study of aging and have proven useful in the identification of genes involved in aging in higher organisms. Therefore, the methods described and claimed in the present application are applicable for the identification of environmental stimuli or genetic modifications that may alter lifespan in yeast and other organisms.

6. Though research into factors that determine the lifespan of animals is in its infancy, there already exists ample scientific evidence that the study of yeast replicative lifespan is relevant to the lifespan of other animals, including vertebrates and mammals. It is true that not all aspects of mammalian aging and lifespan can be studied in simple model systems such as yeast. Combinations of “private” and “public” mechanisms determine the lifespan of eukaryotic organisms. “Public” mechanisms of aging are evolutionarily conserved among disparate organisms; whereas “private” mechanisms of aging are specific to a given organism or a closely related group of organisms” (Smith et al., “Genome-Wide Identification of Conserved Longevity Genes in Yeast and Worms,” *Mechan. Aging and Develop.* 128:106-111 (2007) at 106 (copy attached hereto as **Exhibit 1**)).

7. The best understood public aging phenomenon is caloric restriction (CR). CR, which generally involves moderate reductions in food intake, has been shown to extend the lifespan of yeasts, fruit flies, worms, and mice (Dilova et al., “Calorie Restriction and the Nutrient Sensing Signaling Pathways,” *Cell. Mol Life Sci.* 64(6):752-767 (2007) at 752, 754-756 (“Dilova”) (copy attached hereto as **Exhibit 2**)). CR is an “ancient” phenomenon that evolved in single-cell eukaryotes millions of years before the appearance of metazoan animals. Landmark

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studies on the molecular mechanism of CR in yeast have led to breakthroughs in the understanding of CR in metazoan animals. The existence of genes that regulate lifespan via CR was first demonstrated in yeast, with the discovery that overexpression of the sirtuin protein, Sir2p, extends yeast replicative lifespan (Lin et al., "Requirement of NAD and SIR2 for Life-Span Extension by Caloric Restriction in *Saccharomyces cerevisiae*," *Science* 289:2126-2128 (2000) at 2128 (copy attached hereto as **Exhibit 3**)). Genetic studies have since extended these results to show that sirtuins regulate lifespan in the worm, *C. elegans*, and in the fruit fly, *D. melanogaster* (Haigis et al., "Mammalian Sirtuins—Emerging Roles in Physiology, Aging and Calorie Restriction," *Genes Develop.* 20:2913-2921 (2006) at 2913 (copy attached hereto as **Exhibit 4**)). Therefore, the role of the sirtuins in the lifespan of evolutionarily distinct animals was first discovered in yeast.

8. The pursuit and discovery of small molecules that can delay age-related diseases and increase lifespan in mammals was directed by early studies on the role of yeast Sir2p in replicative lifespan. The study of mammalian Sir2p-like proteins has focused attention on the sirtuins as potential pharmacological targets to treat the major diseases of aging (Porcu and Chiarugi, "The Emerging Therapeutic Potential of Sirtuin-Interacting Drugs: From Cell Death to Lifespan Extension," *Trends Pharm. Sci.* 26(2):94-103 (2005) at 99-101 (copy attached hereto as **Exhibit 5**)). An *in vitro* biochemical screen for small molecules that activate the mammalian Sir2p homolog, SIRT1, led to the finding that the plant compound resveratrol may act as a "caloric restriction mimetic" (Howitz et al., "Small Molecule Activators of Sirtuins Extend *Saccharomyces cerevisiae* Lifespan," *Nature* 425:191-196 (2003) at 192-193 (copy attached hereto as **Exhibit 6**)). Resveratrol increases the lifespan of yeast, fruit flies and a vertebrate fish. Resveratrol also mitigates several deleterious physiological effects of a high-calorie diet in mice (Baur et al., "Resveratrol Improves Health and Survival of Mice on a High-Calorie Diet," *Nature* 444:337-42 (2006) at 337, 338-340 (copy attached hereto as **Exhibit 7**)). A separate study in mice has also demonstrated that resveratrol protects animals against diet-induced obesity and insulin resistance (Lagouge et al., "Resveratrol Improves Mitochondrial Function and Protects Against Metabolic Disease by Activating SIRT1 and PGC-1 α ," *Cell* 127: 1109-1122 (2006) at 1110, 117 (copy attached hereto as **Exhibit 8**)). Other examples of public

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mechanisms of aging are reviewed by Dilova, and include the TOR signaling pathway and glucose-sensing pathways (Dilova at 754-756).

9. The results described in paragraphs 6-8 above prove that the study of yeast replicative lifespan is directly relevant to public aging and senescence mechanisms that are conserved in higher animals. As concluded by Dilova: “Owing to the remarkable evolutionary conservation in longevity regulating proteins and signaling pathways, studies of longevity regulation in model organisms have contributed and will continue to provide a wealth of information relevant to *human* aging and age-associated diseases” (emphasis added). Exhibit 2 at 762. Therefore, the study of yeast replicative lifespan can provide meaningful identification of “public” mechanisms of aging with respect to both environmental and genetic modifications.

The Dead Assay is a Valid Model for the Study of Yeast Replicative Lifespan

10. Replicative lifespan in yeast is defined as the number of daughter cells produced by an individual mother cell before the mother cell undergoes senescence and dies. (Bitterman et al., “Longevity Regulation in *Saccharomyces cerevisiae*: Linking Metabolism, Genome Stability, and Heterochromatin.” *Microbiol. Molec. Biol. Rev.* 67(3):376-399 (2003) at 378 (“Bitterman”)) (copy attached hereto as **Exhibit 9**)). Replicative lifespan is usually expressed as the median lifespan of a cohort of cells. The standard replicative lifespan assay currently in wide use typically involves counting the number of daughter cells produced by 30-60 individual mother cells positioned carefully on an agar plate. The replicative cycles of the mothers are monitored by light microscopy throughout the day. Each new daughter cell is counted and removed by micromanipulation, leaving only the mothers to replicate or die. Depending on the maximum lifespan of the strain, it takes between one to two weeks for a skilled person to complete a set of up to about eight assays, assuming the cells are refrigerated overnight. By example, the replicative lifespan of a yeast strain with an average doubling time of 120 minutes and a maximum lifespan of 50 generations can be completed in ten 10-hour days.

11. The present invention represents the development of a faster, high throughput, and less labor intensive replicative lifespan assay that will greatly facilitate the continued study of aging in yeast. Paragraphs 12, 13, and 14 below describe additional

validation of the present assay, which illustrates its capacity to reproduce the important features of yeast replicative lifespan. This work validates this model for use in identifying both environmental stimuli and genetic modifications which have the potential to alter lifespan.

12. The DeaD assay of the present invention allows the analysis of aging genes by systematic mutagenesis. Examples 1 and 2 of the present invention demonstrate that the DeaD assay reproduces lifespan-shortening deletions of the known aging genes *SIR2* and *SGS1*, respectively. The DeaD assay can be employed in large scale systematic studies aimed at delineating the amino acid residues that are critical for the activities of important aging proteins. As shown in Figure 1 (below), the deletion of the well-known aging gene, *SGS1*, causes reduced replicative lifespan by the DeaD assay (curve *sgs1*). The introduction of a plasmid-borne copy of wild-type *SGS1* into the deletion strain rescues the short lifespan (compare curve *sgs1[SGS1]* with the parental curve). An identical plasmid lacking the *SGS1* gene did not rescue the lifespan defect (not shown). These results demonstrate that the assay is an efficient method for screening among systematically inserted point mutations (genetic changes that alter a single nucleotide or amino acid position) of the *SGS1* gene for those that can or cannot rescue the lifespan of an *sgs1* mutant strain. The generation of large numbers of point mutations in genes is a standard and trivial practice. What is not trivial is the large-scale measurement of replicative lifespan. The method of the present invention can be applied for any gene whose deletion causes a reduction or extension of lifespan, such as *SIR2*. This type of analysis requires as many as one hundred or more lifespan assays, which is highly impractical when using the standard microdissection lifespan assay as described in paragraph 10 but relatively easily performed using the assay of the present invention.

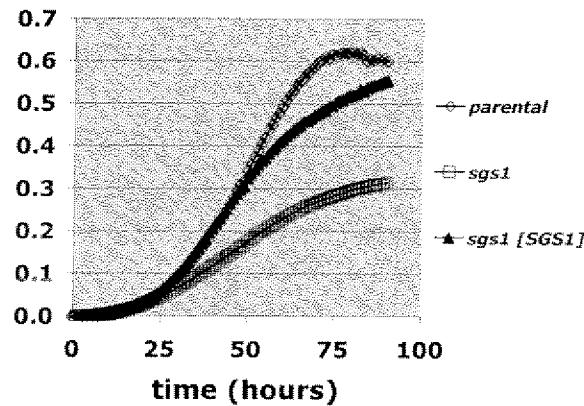


Figure 1. Genetic defects in replicative lifespan can be rescued by introducing a wild-type gene. In this DeaD assay, mutant *sgs1* cells (□) grow poorly compared to parental *SGS1* cells (○) in glucose medium. The introduction of a plasmid encoding wt *SGS1* into the *sgs1* mutant strain (▲) largely rescued the growth (lifespan) defect.

13. One of the most famous results in the aging field was the demonstration that the over-expression of *SIR2* in yeast extends reproductive lifespan. Example 1 of the present application demonstrates that deletion of the *SIR2* gene shortens yeast lifespan as measured using the DeaD assay. As demonstrated herein, over-expression of *SIR2* increases lifespan. In Figure 2 (below) it is shown that yeast replicative lifespan varies in proportion to the level of *SIR2* expression. In this experiment, four different promoters of variable strength were integrated upstream of the *SIR2* chromosomal locus and the replicative lifespan of these four strains, plus a parental strain, were compared by DeaD assay. The under-expression of *SIR2* using the weak CYC promoter reduces lifespan relative to the parental by DeaD assay and standard assay (not shown). The over-expression of *SIR2* using strong TEF and GPD promoters extends lifespan relative to the parental. These results are important for two reasons. First, they show that the DeaD assay reproduces the role of *SIR2* under- and over-expression on lifespan. Second, they show that the DeaD assay is capable of identifying genetic alterations that cause lifespan reduction and/or extension.

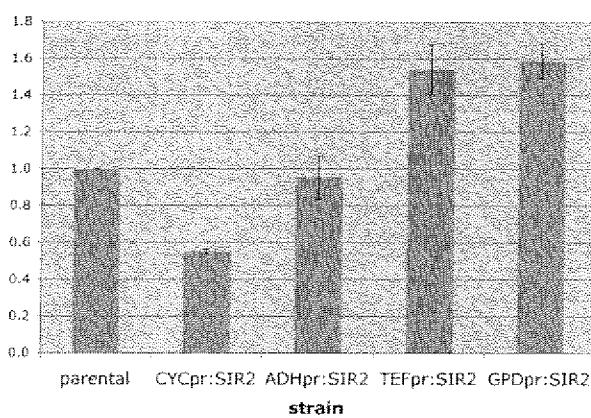


Figure 2. DeaD assay reproduces the relationship between *SIR2* expression level and longevity. Normalized DeaD assay lifespan for five strains containing wild-type *SIR2* or *SIR2* genes expressed using transcriptional promoters of increasing strength (*CYC*, *ADH*, *TEF*, and *GPD*).

14. The DeaD assay of the present invention allows the analysis of environmental factors that influence aging. This is relevant to both “public” mechanisms as well as “private” yeast mechanisms of aging. The experiment described below further validates the

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use of the DeaD assay to assess the affect of various environmental factors. Nicotinamide is a vitamin B₃ precursor known to inhibit Sir2p and shorten replicative lifespan (Anderson et al. "Nicotinamide and PNC1 Govern Lifespan Extension by Caloric Restriction in *Saccharomyces cerevisiae*," *Nature* 423:181-185 (2003) at 181 (attached hereto as **Exhibit 10**)). In Figures 3A-C (below), it is shown that nicotinamide reduces yeast replicative lifespan as measured by the DeaD assay in a dose-dependent fashion without affecting cell growth under permissive conditions (raffinose/galactose).

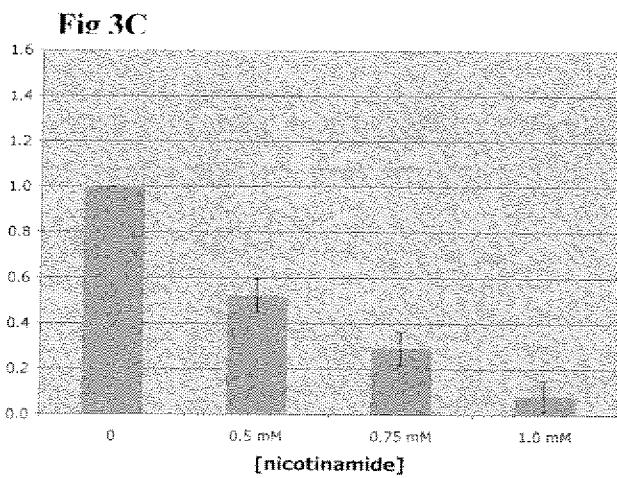
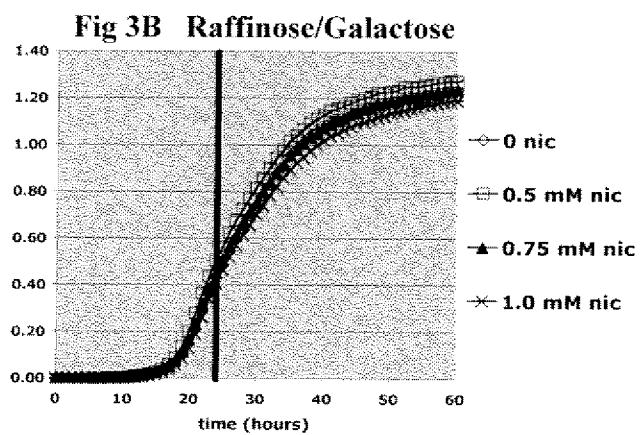
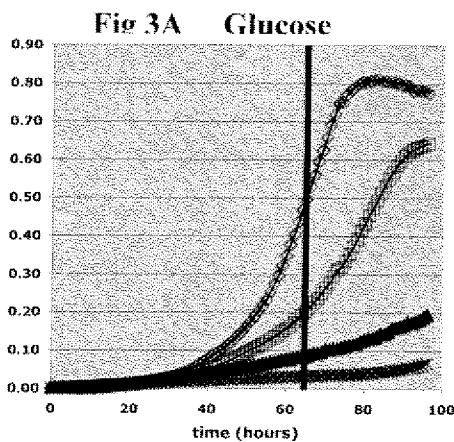


Figure 3A-C. DeaD assay reproduces lifespan reduction by nicotinamide. Fig3A shows that nicotinamide reduces growth in glucose in a dose-dependent fashion. Fig3B demonstrates that these concentrations do not strongly affect growth under permissive conditions. Fig3C shows a strong dose-dependent effect of nicotinamide on replicative lifespan.

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In Figures 4A-B (below), it is shown that the lifespan-shortening affect of nicotinamide is dependent on a wild-type *SIR2* gene. Both of these results reproduce published results using the standard replicative lifespan assay (Bitterman, Exhibit 9, at 389).

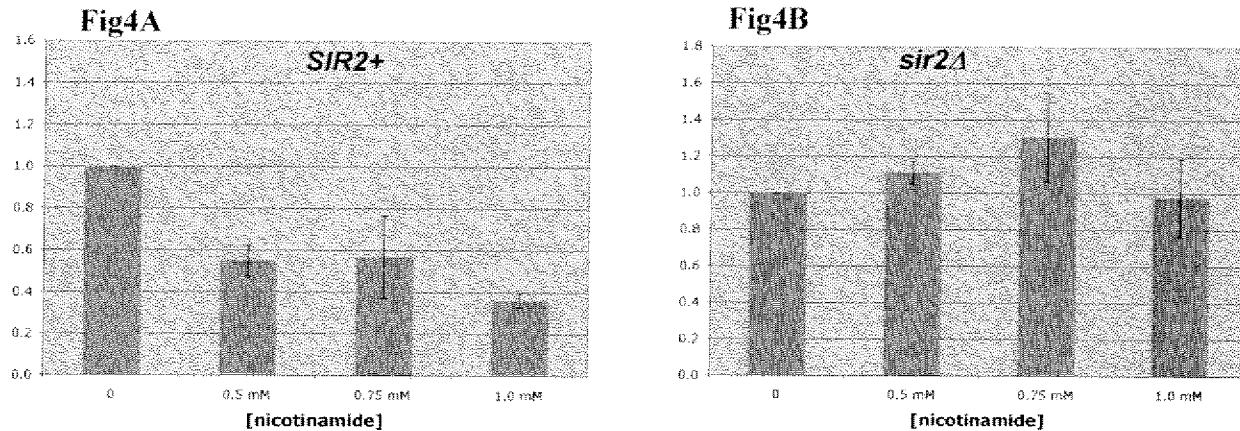


Figure 4A-B DeaD assay reproduces the *SIR2*-dependence of lifespan reduction by nicotinamide. In a DeaD strain lacking the mating type locus HMR, relative lifespans are reduced in a dose-dependent fashion by nicotinamide (Fig4A), but not in cells lacking a *SIR2* gene (Fig4B).

15. Collectively, the validation experiments described in paragraphs 12-14 demonstrate that the assay of the present invention faithfully reproduces results obtained using the standard yeast replicative lifespan assay. More importantly, these results demonstrate that the DeaD assay is sensitive to both genetic modifications, environmental stimuli, and the combination thereof. Therefore, the methods described in the present application are enabling for the study of yeast replicative lifespan and the identification of genetic modifications, environmental stimuli, and the combination thereof that may be relevant to aging in yeast and in higher organisms that share genetically conserved aging pathways.

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16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: May 17, 2007



David S. Goldfarb